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<p><b>(54) Title:</b> INHIBITING TRANSFORMING GROWTH FACTOR <math>\beta</math> TO PREVENT ACCUMULATION OF EXTRACELLULAR MATRIX</p> <p><b>(57) Abstract</b></p> <p>The present invention provides a method for treating or arresting the progress of pathologies characterized by an accumulation of extracellular matrix components by providing an agent to suppress the activity of transforming growth factor <math>\beta</math> (TGF-<math>\beta</math>) a peptide growth factor which is anabolic and leads to fibrosis and angiogenesis. In one embodiment, such agent is anti-TGF-<math>\beta</math> antibody. Pathologies which can be so treated include the various fibrotic diseases. The invention further provides a method for the diagnosis of pathologies, or incipient pathologies, which are characterized by the accumulation of extracellular matrix components in tissues by determining the levels of TGF-<math>\beta</math> in the tissues, a high level being indicative of such pathologies.</p>			

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INHIBITING TRANSFORMING GROWTH FACTOR  $\beta$   
TO PREVENT ACCUMULATION OF EXTRACELLULAR MATRIX

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## BACKGROUND OF INVENTION

This invention relates generally to growth factors and more specifically, to the influence of transforming growth factor- $\beta$  on excessive extracellular matrix production.

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Various pathologies are characterized by a deleterious accumulation of extracellular matrix materials. For example, in progressive glomerular disease, extracellular matrix accumulates in the glomerulus or the glomerular basement membrane, eventually causing end-stage disease and uremia. Similarly, adult respiratory distress syndrome (ARDS) involves the accumulation of matrix materials in the lung while cirrhosis of the liver is characterized by deleterious matrix accumulation in the liver.

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Extracellular matrix is a mixture of proteoglycans, glycoproteins and collagens assembled into a complex superstructure. Although a variety of immunologic, hemodynamic and toxic factors have been used experimentally to induce glomerular disease, none of these factors has been shown to directly influence synthesis or degradation of extracellular matrix components. Thus it seems likely that there is another intervening process between acute cell injury and buildup of glomerular extracellular matrix.

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There thus exists a need to determine the factors which regulate deleterious accumulation of matrix components in pathological states such as kidney disease. Further, there exists a need to control such agents so as to prevent, limit or treat pathogenic conditions which include inappropriate matrix accumulation. The present invention satisfies these needs and provides related advantages as well.

## SUMMARY OF THE INVENTION

The present invention provides a method for treating or arresting the progress of pathologies characterized by 5 an accumulation of extracellular matrix components by providing an agent to suppress the activity of transforming growth factor  $\beta$  (TGF- $\beta$ ), a peptide growth factor which is anabolic and leads to fibrosis and angiogenesis. In one embodiment, such agent is anti-TGF- $\beta$  antibody. Pathologies 10 which can be so treated include the various fibrotic diseases. The invention further provides a method for the diagnosis of pathologies, or incipient pathologies, which are characterized by the accumulation of extracellular matrix components in tissues by determining the levels of 15 TGF- $\beta$  in the tissues, a high level being indicative of such pathologies.

## BRIEF DESCRIPTION OF THE DRAWINGS

20 Figure 1 shows an analysis of dose response effects of TGF- $\beta$  on proteoglycan production by SDS-PAGE. Cultures of rat mesangial cells were treated with TGF- $\beta$  for 48 hours and metabolically labeled with  $^{35}\text{S}$  sulfate. Equal volumes of conditioned media were analyzed by SDS-PAGE and 25 fluorography. Beginning at 0.25 ng/ml (lane 2) there is an increase in PG I and PG II bands. At 2.5 ng/ml (lane 4) a shift in electrophoretic mobility is seen and at higher concentrations of 75 and 150 ng/ml (lanes 7 and 8) an inhibitory effect is present. Lane 1 is control and lanes 30 2-8 represent TGF- $\beta$  at 0.25, 0.75, 2.5, 25, 75 and 150 ng/ml.

Figure 2 shows the effect of TGF- $\beta$  on synthesis of proteins secreted by mesangial cells. Cultures of rat 35 mesangial cells were treated with TGF- $\beta$  for 48 hours and metabolically labeled with  $^{35}\text{S}$  methionine. Equal volumes of conditioned media were analyzed by SDS-PAGE and

fluorography. TGF- $\beta$  did not affect the general pattern of secreted proteins.

Figure 3 shows effects of growth of factors on 5 proteoglycan production. Cultures of rat mesangial cells were treated with growth factors for 48 hours and metabolically labeled with  $^{35}$ S sulfate. Equal volumes of conditioned media were analyzed by SDS-PAGE and fluorography. TGF- $\beta$  increased two broad bands centered at 10 220 kD (biglycan) and 120 kD (decorin) and induced a structural change detected as a shift in electrophoretic mobility. PDGF, IL-1 and TNF had no significant effects.

Figure 4 shows the effect of PDGF on the increased 15 proteoglycan synthesis induced by TGF- $\beta$ . Equal volumes of media from cultures labeled with  $^{35}$ S sulfate and treated with various growth factor combinations were analyzed by SDS-PAGE and fluorography. (Lane 1: control; Lane 2: TGF- $\beta$ , 25 ng/ml; Lane 3: TGF- $\beta$  25 ng/ml + PDGF, 1 U/ml; 20 Lane 4: TGF- $\beta$  25 ng/ml + PDGF, 0.5 U/ml).

Figure 5 shows the immunological identification of 25 proteoglycans affected by TGF- $\beta$ . Equal volumes of control (lanes 1 and 3) or TGF- $\beta$  treated (lanes 2 and 4) conditioned media were immunoprecipitated with antiserum to synthetic peptides of the human core proteins of biglycan (lanes 1 and 2) and decorin (lanes 3 and 4). TGF- $\beta$  specifically increased the biglycan and decorin bands (lanes 2 and 4) compared to control.

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Figure 6 shows the characterization of the 35 proteoglycans regulated by TGF- $\beta$ . Metabolically labeled conditioned media were subjected to specific enzyme digestion. Lanes 1-4 are control and 5-8 are media from TGF- $\beta$  treated cultures (25 ng/ml). Lanes were treated with: saline (1 and 5), heparinase (2 and 6), chondroitinase ABC (3 and 7) and chondroitinase AC (4 and

8). The bands were digested in lanes 3 and 7 indicating the presence of a chondroitin/dermatan sulfate proteoglycan. Note the appearance of a core protein band (lane 7) which has been increased by TGF- $\beta$ .

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Figure 7 shows the extracellular matrix in experimental glomerulonephritis. The percent of glomerular area occupied by extracellular matrix was semiquantitated during the course of glomerulonephritis induced by 10 injection of anti-thymocyte serum (n=30 glomeruli scored in each of 6 animals at each time point). \*p<0.001 \*\*p<0.01 nephritic animals compared to normal control. Values are mean  $\pm$  SD.

15 Figure 8 shows the glomerular ultrastructure in experimental glomerulonephritis. Electron micrographs showing area of normal mesangial matrix (A) in a control animal and an area of increased mesangial matrix (B) in an animal on day 14 of glomerulonephritis induced by injection 20 of anti-thymocyte serum (x6,000).

Figure 9 shows proteoglycan production by cultured glomeruli. Equal numbers of glomeruli isolated from animals (n=2 at each time point) on day 0 (control) or 1, 25 4, 7, 14 and 28 days after injection of anti-thymocyte serum were cultured for 24 hours and biosynthetically labeled with  $^{35}$ S sulfate. Conditioned media was analyzed by SDS-PAGE with fluorography. Compared to day 0, there was a 17-fold increase in biglycan and decorin production on 30 day 4, a 49-fold increase on day 7, a 20-fold increase on day 14 and a 5-fold increase on day 28.

Figure 10 shows the effect of conditioned media from nephritic glomeruli on proteoglycan production by normal 35 cultured mesangial cells. The cells were biosynthetically labeled and the conditioned media analyzed by SDS-PAGE with fluorography. The conditioned media from nephritic

glomeruli stimulated the production of biglycan and decorin beginning on day 1, peaking on day 7 and then production decreased toward control levels by day 28.

5       Figure 11 shows the effect of anti-TGF- $\beta$  synthetic peptide antibody on stimulation of proteoglycan production by conditioned media from nephritic glomeruli. Anti-TGF- $\beta$  antibody (Ab) or normal preimmune serum (NS) was mixed with the conditioned media from nephritic glomeruli isolated on 10 day 4 (GN 4) and 7 (GN 7) following injection of anti-thymocyte serum. The antibody reduced proteoglycan production by 77 percent (GN 4) and 68 percent (GN 7).

15       Figure 12 shows the specificity of the blocking effect of the anti-TGF- $\beta$  antibody. Conditioned media from nephritic glomeruli on day 7 following anti-thymocyte serum injection were mixed with normal preimmune serum (NS), anti-TGF- $\beta$  antibody (AB), or immunizing peptide (P) plus antibody. The peptide abolished the ability of the 20 antibody to block the stimulation of proteoglycan production.

25       Figure 13 shows enzymatic identification of the proteoglycans induced by conditioned media from nephritic glomeruli on day 7 following anti-thymocyte serum injection. Lane 1 is a control treated with saline. Lanes were treated with: heparinase II (lane 2), chondroitinase ABC (lane 3) and chondroitinase AC (lane 4). Complete digestion of the 220 KD and 120 KD bands is seen in lane 3 30 and partial digestion in lane 4, indicating the presence of chondroitin/dermatan sulfate proteoglycans.

35       Figure 14 shows the immunological identification of the proteoglycans from the conditioned media shown in Figure 13. Equal volumes of conditioned media from control or nephritic glomeruli were immunoprecipitated with antiserum to synthetic peptides of the human core protein

of biglycan (lanes 1 and 2) and decorin (lanes 3 and 4). The biglycan (lane 2) and decorin (lane 4) bands were specifically increased in the conditioned media from the nephritic glomeruli (lanes 2 and 4) compared to control 5 (lanes 1 and 3).

Figure 15 shows the expression of TGF- $\beta$  in the kidney. Glomerular cells synthesizing TGF- $\beta$  after injection of anti-thymocyte serum were detected by immunofluorescence 10 (n=30 glomeruli counted in each of 6 animals at each time point). \*p<0.001 nephritic animals compared to normal control. Values are mean  $\pm$  SD.

Figure 16 shows immunofluorescence micrographs of 15 glomeruli stained with anti-TGF- $\beta$  antibody. There is a striking increase in the number of glomerular cells staining for TGF- $\beta$  on day 7 (B) following induction of glomerulonephritis, compared to control (A) (x500).

20 Figure 17 shows micrographs showing the enlargement of glomeruli in nephritic kidneys. Kidneys from rats made nephritic by an injection of anti-thymocyte serum and examined on day 14 after the injection. Panel A is from a rat that received normal rabbit serum injections four 25 successive days, starting on the day of the anti-thymocyte serum injection. Panel B is from a rat that received rabbit anti-TGF- $\beta$  under a similar regimen. Toluidine blue staining. X500 magnification

30 Figure 18 shows proteoglycan synthesis by glomeruli from nephritic rats treated with TGF- $\beta$ . Glomeruli were isolated 4 (GN4) and 7 (GN7) days after the injection of the anti-thymocyte serum that has been followed by treatments similar to those described in the legend of 35 Figure 17, and placed in culture. Proteoglycan synthesis was examined by labeling the cultures with  $^{35}\text{SO}_4$  followed by analysis of the secreted products by SDS-PAGE and

autoradiography. N3, nephritic rats treated with normal rabbit serum  $\alpha$ TGF- $\beta$ ; IS nephritic rats treated with rabbit anti-TGF- $\beta$ . The control lane (N) shows proteoglycan production in glomeruli from a normal kidney and the 5 positions of molecular weight markers are indicated to the left.

DETAILED DESCRIPTION OF THE INVENTION

10 The invention provides a method for treating pathologies characterized by an accumulation of extracellular matrix in a tissue, comprising contacting the tissue with an agent which suppresses the extracellular matrix producing activity of TGF- $\beta$ . The agent can be anti-  
15 TGF- $\beta$  antibody, PDGF or an Arg-Gly-Asp-containing peptide. Preferably, such Arg-Gly-Asp containing peptide is between 4 and 50 amino acids in length.

20 The pathologies can be selected from the group consisting of glomerulonephritis, adult respiratory distress syndrome, cirrhosis of the liver fibrotic cancer, fibrosis of the lungs, arteriosclerosis, post myocardial infarction, cardiac fibrosis, post-angioplasty restenosis, renal interstitial fibrosis and scarring.

25 The invention also provides method of inhibiting the accumulation of extracellular matrix in a tissue, comprising suppressing the activity of TGF- $\beta$  in the tissue. Suppressing the activity of TGF- $\beta$  comprises contacting the 30 tissue can be with anti-TGF- $\beta$  antibodies, PDGF or an Arg-Gly-Asp-containing peptide. The tissue can be comprised of cells selected from the group consisting of kidney, lung, liver and skin cells.

35 A method is also provided of detecting the presence of pathologies of a tissue characterized by an excessive accumulation of extracellular matrix components, comprising

determining the level of TGF- $\beta$  in said tissue and comparing the level of TGF- $\beta$  in the tissue to the level of TGF- $\beta$  in normal tissues, an elevated level of TGF- $\beta$  in the tissue being indicative of such pathologies. The pathologies can 5 be selected from the group consisting of glomerulonephritis, adult respiratory distress syndrome, cirrhosis of the liver, fibrotic cancer, fibrosis of the lungs, arteriosclerosis, post myocardial infarction, cardiac fibrosis, post-angioplasty restenosis, renal 10 interstitial fibrosis and scarring.

A method is also provided of decreasing the production of an extracellular matrix component by a cell which produces an extracellular matrix component comprising 15 decreasing the amount or inhibiting the activity of TGF- $\beta$  to which said cell is exposed. The cell can be a mesangial cell or an epithelial cell. The extracellular matrix component can be a proteoglycan. The proteoglycan can be biglycan or decorin.

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The extracellular matrix component can be fibronectin, laminin and type IV collagen.

Also provided is an antibody which inhibits the 25 proteoglycan stimulating activity of TGF- $\beta$  having an affinity of about  $10^8$  or greater and a titer of about 1:30,000 or greater as measured by radio immunoassay. The antibody can be produced by immunizing an animal with a linear peptide from TGF- $\beta$ . A cell which produces the 30 antibody is also provided.

As noted, the invention provides a method of inhibiting the accumulation of extracellular matrix in a tissue by suppressing the activity of TGF- $\beta$  in the tissue. 35 Also provided is a method for treating pathologies characterized by an accumulation of extracellular matrix in a tissue by suppressing the activity of TGF- $\beta$ . TGF- $\beta$  is

comprised of two chains each of 112 amino acids. TGF- $\beta$  is responsible for the increased synthesis of extracellular matrix observed in various pathologies. The amino acid sequence of TGF- $\beta$  is as follows:

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Arg His Arg Arg Ala Leu Asp Thr Asn Tyr Cys Phe Ser Ser Thr  
Glu Lys Asn Cys Cys Val Arg Gln Leu Tyr Ile Asp Phe Arg Lys  
Asp Leu Gly Trp Lys Trp Ile His Glu Pro Lys Gly Tyr His Ala  
Asn Phe Cys Leu Gly Pro Cys Pro Tyr Ile Trp Ser Leu Leu Asp  
10 Thr Gln Tyr Ser Lys Val Leu Ala Leu Tyr Asn Gln His Asn Pro  
Gly Ala Ser Ala Ala Pro Cys Cys Val Pro Gln Ala Leu Glu Pro  
Leu Pro Ile Val Tyr Tyr Val Gly Arg Lys Pro Lys Val Glu Gln  
Leu Ser Asn Met Ile Val Arg Ser Cys Lys Cys Ser

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A variety of growth factors have been suggested to play a role in extracellular matrix production. However, their influence on the pathological accumulation of matrix components has been unclear. The invention is predicated on the new discovery that tissues prone to pathological 20 accumulation of matrix synthesize particular proteoglycans. Agents which inhibit TGF- $\beta$  activity, such as antibodies reactive with TGF- $\beta$ , have been found to block the stimulatory effect of TGF- $\beta$  on proteoglycan production. In this respect, TGF- $\beta$  is unique among growth factors 25 tested, and thus manipulating this specific effect of TGF- $\beta$  has utility in controlling or treating the inappropriate and undesirable accumulation of matrix components in various pathologies.

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Mesangial cells are one of the cell types that make up kidney glomerulus. In the normal glomerulus, the mesangial cells are surrounded by extracellular matrix. An increase in the quantity of mesangial matrix, with or without mesangial hypercellularity, is the earliest histologic 35 finding in many forms of glomerulonephritis and in diabetic nephropathy. Cultured mesangial cells are known to secrete several matrix components including proteoglycans,

fibronectin, laminin, entactin, thrombospondin and collagen types I, III, IV and V. However, the exact composition and supramolecular organization of the mesangial matrix, as well as the factors that control its synthesis assembly and 5 degradation, have been unknown.

To study factors controlling the composition of the mesangial matrix, mesangial cells in culture were treated with IL-1, PDGF, TNF and TGF- $\beta$ . Analysis of the culture 10 media indicated that TGF- $\beta$  increased the amount of two components, identified as the proteoglycans biglycan and decorin. PDGF, IL-1, and TNF had no significant effect over the control.

15 Glomerulonephritis can be induced by specific immunological injury to the mesangial cell. Glomeruli isolated show increased biglycan and decorin production. Moreover, conditioned media from cultured nephritic mesangial cells stimulate biglycan and decorin synthesis by 20 normal mesangial cells. An equivalent stimulatory effect can be produced by the addition of exogenous TGF- $\beta$ . Moreover, agents which can block the effect of TGF- $\beta$ , such as an antiserum, block the stimulatory effect of exogenous TGF- $\beta$ . Such agents, including monoclonal or polyclonal 25 antibodies, PDGF and Arg-Gly-Asp containing peptides, can be used to specifically control or treat deleterious matrix proteoglycan synthesis. Thus, such agents can be used to prevent any condition associated with extracellular matrix accumulation, for example scaring, or to treat pathologies 30 characterized by an accumulation of extracellular matrix in a tissue by contacting the tissue with an agent which suppresses TGF- $\beta$  activity. The pathologies capable of treatment are characterized by an accumulation of extracellular matrix. These diseases are generally 35 fibrotic diseases and include glomerulonephritis, adult respiratory distress syndrome and cirrhosis of the liver. Also included are fibrocytic disease, fibrosclerosis and

fibrosis, as well as fibrotic cancers, such as of the breast, uterus or pancreas, including fibroids, fibroma, fibroadenomas and fibrosarcomas. In addition, the method can be used to treat such fibrotic conditions as fibrosis 5 of the lungs, arteriosclerosis, post myocardial infarction, cardiac fibrosis and post-angioplasty restenosis and renal interstitial fibrosis. In addition, the method can be used to treat or prevent excessive scarring such as keloid scars. However, these pathologies are merely 10 representative and a person skilled in the art would readily recognize the method to be useful in any pathology associated with accumulation of extracellular matrix.

The presence of elevated levels of TGF- $\beta$  can be used 15 diagnostically to determine the presence or incipient presence of pathologies deriving from extracellular matrix accumulation. For example, immunoassays utilizing anti-TGF- $\beta$  antibodies provide such a diagnostic test. Various formats of such assays are available and well known to 20 those skilled in the art, including RIA, ELISA and immunofluorescence. See generally, Ruoslahti et al., M. Enz., 82:803-831 (1982) which is incorporated by reference herein. Alternatively, nucleic acid probes can be used to detect and quantitate TGF- $\beta$  mRNA for the same purpose. 25 Such methods are also well known in the art.

Additionally, a method of decreasing the production of a proteoglycan by a cell which produces a proteoglycan is provided. The method comprises decreasing the amount of 30 TGF- $\beta$  to which the cell is exposed. Such amount of TGF- $\beta$  can readily be ascertained, i.e. an amount under that present in a normal cell leading to decreased proteoglycan production. Alternatively, the cellular production of a 35 proteoglycan can be decreased by inhibiting the proteoglycan producing activity of TGF- $\beta$ . This inhibition can be performed by the methods taught in this invention, for example, binding TGF- $\beta$  with a ligand. Additionally, it

is recognized that certain modifications or amino acid substitutions can be performed on TGF- $\beta$  without changing its essential function. Thus, by "TGF- $\beta$ " is meant all modifications to TGF- $\beta$  as long as the essential function of 5 increasing extracellular matrix production is maintained. TGF- $\beta$ 1 and TGF- $\beta$ 2 both exhibit this function, see for example Ignatz and Massague, J. Biol. Chem. 261:4337-4345 (1986) and Bassols and Massague, J. Biol. Chem. 263:3083-3095 (1986) both of which are incorporated by reference 10 herein.

The following examples are intended to illustrate but not limit the invention.

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#### EXAMPLE I

##### EFFECT OF TGF- $\beta$ ON MESANGIAL CELL CULTURE

Mesangial cells were obtained from intact glomeruli of 20 4 to 6 week old Sprague-Dawley rats according to the method of Harper, et al., Kidney International 26:875 (1984), which is incorporated herein by reference. The growth medium used was RPMI 1640 (Cell-Gro, Washington, D.C.) supplemented with 20% heat-inactivated fetal calf serum 25 (FCS) (Hyclone, Logan, UT), 50 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.66 U/ml insulin, and 300 mg/ml L-glutamine. Between day 15 to 20, primary cultures were detached with a solution of 0.025% trypsin - 0.5 mM EDTA (Flow Labs, McLean, VA) and  $2 \times 10^6$  cells were added to flasks. The 30 cells were passed every 7 days and all experiments were performed on cells between passages 3 and 7.

Phase contrast and immunofluorescence microscopy was performed by growing cells to subconfluence on coverslips 35 in plastic wells. The cells were fixed with 3.7% paraformaldehyde for 10 minutes at 22°C. After washing with phosphate buffered saline (PBS), the cells were

incubated with specific antibodies and washed and reincubated with FITC-anti-rabbit IgG. The coverslips were mounted and examined by phase contrast and immunofluorescence microscopy. This technique revealed a 5 homogeneous population of cells that were identified as mesangial cells according to the following findings: 1) presence of spindle shaped morphology, 2) absence of polygonal-shaped cells, 3) bright immunofluorescence staining for myosin, actin, desmin, and anti-thy 1.1 10 antibody and negative staining for common leukocyte antigen, cytokeratin and factor VIII. The cells also showed no morphologic evidence of toxicity upon exposure to aminonucleoside of puromycin (Sigma, St. Louis, MO).

15 To study proteoglycan synthesis, equal numbers of cells were added to 6-well multiwell plates or petri dishes and allowed to grow to subconfluence. Cultures were made serum free for 24 hours to arrest cell proliferation. The cell layers were washed 3 times with sterile PBS and serum 20 and antibiotic free RPMI was added as a low sulfate growth medium for <sup>35</sup>S methionine labeling. The following growth factors were added to the media for 48 hours: TGF- $\beta$ , bovine or porcine (R&D Systems, Inc., Minneapolis, MN) human PBGF (Collaborative Research, Inc., Bedford, MA) 25 human recombinant IL-1  $\alpha$  (Collaborative Research, Inc. Bedford, MA) and recombinant human TNF (Amgen, Thousand Oaks, CA). The concentrations chosen were: TGF- $\beta$  (25 ng/ml), PDGF (10 U/ml), IL-1 (5 U/ml) and TNF (500 U/ml). Eighteen hours prior to termination of the experiment, <sup>35</sup>S 30 methionine (150  $\mu$ Ci/ml), to label proteins, or <sup>35</sup>S sulfate (200  $\mu$ Ci/ml), to label proteoglycans, were added to the cultures. Isotopes were obtained from New England Nuclear (Boston, MA). The culture media were removed, phenylmethylsulfonyl fluoride (PMSF), pepstatin and 35 aprotinin (Sigma, St. Louis, MO) were added to protease inhibitors, and the mixtures were centrifuged for 20 minutes to remove debris. The remaining cell monolayers

were removed by washing 2 times with PBS followed by incubation with 300  $\mu$ l of SDS-PAGE sample buffer. The layers were detached by agitation. Samples were electrophoresed immediately and the remainder stored at -5 20°C.

The uptake of  $^3$ H-thymidine as a marker of cell proliferation was evaluated by the addition of 10  $\mu$ l per well of a 1 mCi/ml solution of  $^3$ H-thymidine (84 curies/ $\mu$ Mol) 10 diluted 1:100 with sterile PBS. The incubations were carried out for 24 or 48 hours after which the cells were harvested onto glass fiber filter mats using a cell harvester (Skatron, Lierbyen, Norway). Prior to harvesting the cells, the media was aspirated and the cells were 15 washed twice with Hank's balanced salt solution. Incorporation of  $^3$ H-thymidine into cellular DNA was measured by counting the filter mats in a liquid scintillation counter (Beckman, Irvine, CA). In separate experiments aliquots of cells were counted visually in a hemacytometer 20 to verify that the incorporation of  $^3$ H-thymidine paralleled changes in cell numbers. Prior to harvesting, cells were evaluated for evidence of cytotoxicity by phase microscopy. Cell viability was also assessed by Trypan blue exclusion.

25 The biosynthetic labeling of cultured mesangial cells with  $^{35}$ S sulfate, to label proteoglycans, and  $^{35}$ S methionine, to label proteins, showed that the addition of TGF- $\beta$  induced a dramatic increase in the production of proteoglycans. Under control conditions, the mesangial 30 cells secreted into the medium two distinct small proteoglycans that were identified as broad bands on SDS-PAGE, centered at 220 and 120 kD (Figure 1). In addition, there was some labeled material that did not enter the gel. The intensity of each of these bands was increased by the 35 TGF- $\beta$  treatment and the bands stopped at a slightly higher molecular weight. The maximal effect of TGF- $\beta$  occurred at 25 ng/ml where there was an 8 to 10 fold increase in

proteoglycan production compared to control levels. At higher concentrations there was a decrease in the action of TGF- $\beta$  with the effects on proteoglycan production disappearing at 150 ng/ml. Examination of proteoglycan 5 incorporation into the extracellular matrix by extraction and parallel analysis of the cell layer, showed a qualitatively identical TGF- $\beta$  effect. However, the bands were considerably less intense indicating that the proteoglycans were mostly secreted into the medium. Under 10 the experimental conditions employed, no demonstrable effect on the pattern of protein synthesis was observed, as revealed by  $^{35}$ S methionine labeling followed by SDS-PAGE (Figure 2) and analysis of the gels by laser densitometry. None of the other growth factors, PGDF, IL-1 or TNF, caused 15 induction of proteoglycans similar to TGF- $\beta$  (Figure 3).

In order to study potential growth factor interactions, mesangial cells were exposed to IL-1, PDGF and TNF before adding TGF- $\beta$  as described above. None of 20 these three growth factors alone altered proteoglycan production. PDGF, however, when added to the cells before TGF- $\beta$ , blocked the expected increase in proteoglycan production (Figure 4). The blocking effect did not occur with IL-1 or TNF. The peptide GRGDSP also blocked the 25 increased proteoglycan synthesis caused by the addition of TGF- $\beta$  to the mesangial cell cultures, whereas peptide GRGESP did not (Pierschbacher and Ruoslahti, Nature 309:30-33 (1984), which is incorporated herein by reference).

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## EXAMPLE II

IDENTIFICATION OF PROTEOGLYCAN SPECIES

## 1. Immunoprecipitation

35 Immunoprecipitations were performed by adding 100  $\mu$ l of antiserum to 500  $\mu$ l of conditioned medium or 300  $\mu$ l of cell extract collected from duplicate wells in the presence

or absence of added growth factors. In duplicate wells, cells were detached and counted to ensure uniformity of cell number. Preimmune serum was used in parallel control experiments. The samples were incubated overnight at 4°C 5 with mixing in 4 ml conical tubes precoated with bovine serum albumin (BSA). Protein-A-Sepharose beads (Sigma, St. Louis, MO) were preincubated with fresh RPMI for 60 minutes at 22°C. To precipitate the antigen-antibody complexes, 50  $\mu$ l of suspended protein-A-Sepharose was added to the 10 samples, and mixed at 4°C for 120 minutes. The samples were centrifuged for 10 minutes at 2000  $\times$  G and the supernatant removed. The pellets were washed 10 times with 1 ml of ice cold PBS containing 0.5 M NaCl, 0.1% Triton X-15 100 pH 7.4. Finally, the pellets were washed with ice cold PBS, transferred to new tubes, re-centrifuged, and washed 3 times with PBS. The pellets were dissolved in 40  $\mu$ l of SDS-PAGE sample buffer containing 3% SDS and 10%  $\beta$  mercaptoethanol (Sigma, St. Louis, MO) and boiled for 5 minutes.

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The molecular size and type of the two proteoglycans produced by mesangial cells and regulated by TGF- $\beta$ , corresponded to that of two proteoglycans, biglycan (PG I) and decorin (PG II). These proteoglycans are known to have 25 45 kD core proteins, the sequences of which have been deduced from cDNA (Krusius, T and Ruoslahti, E., Proc. Natl. Acad. Sci., USA 83:7683-7687 (1986) and Fisher et al., J. Biol. Chem. 264:4571-4576 (1989) which are incorporated herein by reference). Polyclonal antibodies 30 produced to synthetic peptides from the N-terminal sequences of these proteoglycans were used to identify the proteoglycans in the mesangial cell culture media. Immunoprecipitation of  $^{35}$ S sulfate labeled conditioned media from control and TGF- $\beta$  treated cells followed by SDS-PAGE 35 identified the 220 kD band as biglycan and the 120 kD band as decorin (Figure 5). Immunoprecipitation of conditioned media from  $^{35}$ S methionine-labeled cells with anti-

fibronectin, laminin and type IV collagen antibodies and SDS-PAGE analysis of the immunoprecipitates showed no visible effect of TGF- $\beta$  on the levels of these proteins (data not shown).

5

## 2. Enzyme Digestion

Digestion with glycosaminoglycan-degrading enzymes was used to determine the type of proteoglycans that were 10 regulated by TGF- $\beta$ . The digestion was performed on conditioned media after biosynthetic labeling. Aliquots of medium (25 $\mu$ l) were mixed with 100 milliunits of chondroitinase ABC or chondroitinase AC both in 100 mM Tris-HCl, pH 7.5, 10 mM calcium acetate, 2 mg/ml BSA or 100 15 milliunits of heparinase II in 50 mM Tris-HCl, pH 7.4, 1 mM calcium chloride, 5 mM calcium acetate. All samples also received 1 mM PMSF, 5 mM benzamidine, 100  $\mu$ g/ml soy bean trypsin inhibitor, 10  $\mu$ g/ml leupeptin and 10  $\mu$ g/ml antipain. All materials were obtained from Sigma. 20 Chondroitinase-containing mixtures were incubated at 37°C for 1.5 hours. At termination samples were prepared for SDS-PAGE.

The small proteoglycans from control and TGF- $\beta$  treated 25 cells were degraded by chondroitinase ABC but were insensitive to chondroitinase AC and heparinase (Figure 6). The labeled material (large proteoglycan(s)) at the top of the gel appeared to be partially sensitive to digestion with both chondroitinase ABC and heparinase. These results 30 indicate that the major proteoglycans produced by mesangial cells are chondroitin/dermatan sulfate proteoglycans. In addition to identifying the glycosaminoglycan type, incubation of TGF- $\beta$  conditioned medium with chondroitinase ABC resulted in the appearance of a new 45 kD band (Figure 35 6). This band is likely to represent the proteoglycan core proteins after removal of portions of the chondroitin/dermatan sulfate chains. Enzyme treatment of

conditioned medium from control cells did not yield a visible core protein. This indicates that part of the regulatory action of TGF- $\beta$  is to stimulate new synthesis of the proteoglycan core proteins.

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### EXAMPLE III

#### INDUCTION OF EXPERIMENTAL GLOMERULONEPHRITIS,

#### HISTOLOGIC EXAMINATION, PREPARATION OF

#### GLOMERULAR CULTURES, CULTURE MEDIA

10

#### AND ANTIBODY PRODUCTION

##### a. Induction of Experimental Glomerulonephritis

To study the role of TGF- $\beta$  in the glomerular 15 proteoglycan synthesis in vivo, a glomerulonephritis model in which the disease is induced with an anti-thymocyte serum (ATS) was produced by immunizing New Zealand white rabbits with  $1 \times 10^6$  rat thymocytes in complete Freund's adjuvant, followed by boosting with  $1 \times 10^6$  thymocytes given 20 intravenously two and four weeks later. Preimmunization serum was collected from the same animal and used in control experiments as normal rabbit serum. Prior to use, ATS and normal serum were absorbed 3 times each with packed rat erythrocytes and rat liver powder. The serum was then 25 heat inactivated at 56 °C for 30 minutes. Glomerulonephritis was induced in Sprague Dawley rats (4-6 weeks old) by intravenous administration of 1 ml ATS per 100 g body weight and 1 ml normal rabbit serum as a source of complement. Control animals received an equal volume of 30 normal serum instead of ATS. Animals were sacrificed on days 1, 4, 7, 14 and 28 following ATS administration for histologic examination of kidney tissue and isolation of glomeruli for culture. On the day of sacrifice, systolic 35 blood pressure was measured in the conscious state with a tail-cuff sphygmomanometer (Narco Biosystems, Houston, TX) connected to a recorder (Pharmacia, Uppsala, Sweden) and serum creatinine determined by using Sigma Diagnostics

Creatinine reagents (Sigma, St. Louis, MO). Animals were housed in the metabolic cages and total urine output was collected daily during the first week and weekly thereafter for measurement of 24-hour protein excretion by 5 sulfosalicylic acid precipitation according to the standard methods. See for example, Border, et al., Kidney International 8:140 (1975), which is incorporated herein.

b. Histologic Examination

10

Kidney tissue from each animal was processed and examined by light, immunofluorescence and electron microscopy as described in Border, et al., Kidney Int. 8:140-148 (1975), which is incorporated herein by 15 reference. For light microscopy, tissues were fixed neutral formalin, embedded in paraffin and  $2\mu$  sections stained with periodic acid-Schiff. To quantitate mesangial matrix and glomerular cellularity, all sections were coded and read by an observer of the experimental protocol 20 applied. Thirty glomeruli (80-100 $\mu$ m in diameter) were selected at random and cell nuclei counted and the degree of glomerular matrix expansion determined using a published method (Raij, et al., Kidney Int. 26:137-143 (1984), which is incorporated herein by reference). Briefly, sections of 25 the kidney are observed under 40x microscopic objective and the percentage of glomerular space occupied by mesangial matrix was estimated and assigned a score beginning with 1=0 to 25%, 2=25 to 50%, 3=50 to 75% and 4=75 to 100%.

30

Immunofluorescence microscopy was performed on tissue snap-frozen in liquid nitrogen, fixed in acetone, and 4  $\mu$ m sections stained with fluorescein isothiocyanate-conjugated antisera (Cooper Biomedical, Malvern, PA) to rabbit and at IgG and C3. For electron microscopy, tissue was placed in 35 Karnovsky's fixative at 4°C overnight, embedded in Epon and ultrathin sections stained with uranyl acetate and lead citrate.

The dose of ATS administered produced an acute form of mesangial injury glomerulonephritis. There was a definite increase in the mesangial extracellular matrix, beginning on day 7, becoming maximal on day 14, and 5 decreasing thereafter (Figure 7). The decrease in matrix noted on days 1 and 4 coincided with a decrease in glomerular cellularity due to complement-mediated lysis of a portion of the mesangial cells. Ultrastructural examination confirmed the increase in mesangial matrix 10 (Figure 8). Functional changes in this model of glomerulonephritis consisted of: 1) transient proteinuria during the first week, 2) no significant change in levels of serum creatinine and, 3) a slight but significant elevation of systolic blood pressure only on day 14 in the 15 nephritic group.

#### c. Glomerular Culture

20 Rats were anesthetized intramuscularly with ketamine HCl, 10 mg/100 g body weight, and xylazine 0.5 mg/100 g body weight. The kidneys were perfused in situ via the aorta with phosphate buffered solution (PBS) (pH 7.4), and then excised. The capsules were removed and the cortical 25 tissue dissected out and minced with a razor blade. Glomeruli were isolated using the graded sieving technique. A spatula was used to pass minced cortex through a 149  $\mu$ m nylon screen (Spectrum, Los Angeles, CA). The tissue which emerged was passed sequentially through a 105  $\mu$ m and 74  $\mu$ m 30 sieve. Intact glomeruli retained on the 74  $\mu$ m sieve were removed, and washed three times in PBS, pH 7.4 and resuspended at  $5 \times 10^3$  glomeruli per ml in serum-free and antibiotic-free RPMI 1640 (Cell-Gro, Washington, D.C.) in 6-well multiwell plates. After 24 hours of incubation, the 35 cultures were biosynthetically labeled by addition of 200  $\mu$ Ci/ml of  $^{35}$ S sulfate for an additional 24 hours. All isotopes were obtained from New England Nuclear (Boston,

MA). The culture media were removed, phenylmethylsulfonyl fluoride, peptain and aprotinin (Sigma, St. Louis, MO) were added as protease inhibitors, and the mixtures centrifuged for 20 minutes to remove cellular debris. Samples were 5 electrophoresed immediately and the remainder stored at -20°C.

d. Preparation of Conditioned Media From Glomerular Cultures

10

Media conditioned by exposure to normal ATS glomeruli for 48 hours was collected. In order to activate precursor TGF- $\beta$ , aliquots of the conditioned media were acidified to pH 3.2 for 1 hour by addition of 1N HCl. The transiently 15 acidified media was brought to pH 7.4 with 1N NaOH and dialyzed against serum-free RPMI for 24 hours at 4°C. In some experiments 100  $\mu$ l of antiserum made against a synthetic peptide from TGF- $\beta$  was added to 1 ml of activated conditioned media and incubated overnight at 4°C with 20 continuous mixing. To determine the specificity of the TGF- $\beta$  antiserum, 100  $\mu$ g of synthetic peptide that had been used for the immunization was added to 1 ml of antiserum and incubated for 2 hours at 22°C with continuous mixing. Prior to addition to mesangial cell cultures, all 25 conditioned media were centrifuged 1000 X G for 20 minutes and passed through a 0.2  $\mu$ m Uniflo filter (Schleicher & Schell, Inc., Keene, NH).

e. Anti-TGF- $\beta$  Antibodies

30

The anti-TGF- $\beta$  antiserum was prepared against a synthetic peptide from residues 78-109 of the human mature TGF- $\beta$ . A partial amino-acid sequence of the human mature form of TGF- $\beta$  is described in Derynck et al., Nature 35 316:701 (1985) which is incorporated by reference herein. Antisera raised against the same peptide, whose terminal cysteine residues were disulfide-linked, have previously

been shown to inhibit the binding of TGF- $\beta$  to its receptors (Flanders et al., Biochemistry 27:739 (1988), which is incorporated herein by reference). The peptide was synthesized in a solid phase peptide synthesizer (Applied 5 Biosystems, Foster City, CA) and purified by HPLC. A rabbit was immunized with 2 mg per injection of the peptide mixed with 0.5 mg of methylated BSA (Benoit et al., Proc. Natl. Acad. Sci. USA 79:917 (1982), which is incorporated herein by reference) and emulsified in Freund's complete 10 adjuvant. The injections were generally given four weeks apart and the rabbit was bled approximately a week after the second and every successive, injection. The bleedings used in this work had a titer (50% binding) of about 1:3,000 to about 1:30,000 in radio immunoassay, bound to 15 TGF- $\beta$ 1 in immunoblots and inhibited the induction of proteoglycan synthesis caused by TGF- $\beta$ 1 in cultured mesangial cells. Further, it is expected that the antibodies would also inhibit TGF- $\beta$ 2. Additionally, the antibody has an affinity of about  $10^8$  or greater as measured 20 by radio immunoassay and calculated as described in Muller, J. Imm. Met. 34:345-352 (1980). Preferably the antibody has an affinity of  $10^9$  or greater. A second polyclonal antibody (anti-LC) made against a synthetic peptide corresponding to the NH<sub>2</sub>-terminal 30 amino acids of mature 25 TGF- $\beta$ .

## EXAMPLE IV

GLOMERULAR PROTEOGLYCAN PRODUCTION AND TGF- $\beta$ 

30 Groups of nephritic animals were sacrificed 1, 4, 7, 14 and 28 days after being injected with ATS. Their glomeruli were isolated, placed in culture, and biosynthetically labeled to identify newly synthesized proteoglycans. One day after ATS injection, proteoglycan 35 synthesis was the same as in normal controls; however, on day 4 there was a striking induction of proteoglycan production, that reached a 49-fold increase on day 7, and

which then declined on days 14 and 28 (Figure 9). To determine if TGF- $\beta$  might be the factor in the conditioned media responsible for the induction of proteoglycan synthesis, the media was transiently acidified to activate 5 TGF- $\beta$ , and then added to normal cultured mesangial cells. The ability to stimulate proteoglycan production is a relatively specific property of TGF- $\beta$  (Bassolis, A. and Massague, J., J. Biol. Chem. 263:3039-3045 (1988), which is incorporated herein by reference); thus, the response of 10 the mesangial cell cultures to the conditioned media can be considered as a bioassay for TGF- $\beta$ . Activated conditioned media from the nephritic glomeruli strongly stimulated proteoglycan production by normal mesangial cells (Figure 10). The temporal pattern of proteoglycan 15 synthesis induced by the conditioned media resembled the proteoglycan production seen in the glomerular cultures (compare Figures 9 and 10). Conditioned media that was not transiently acidified did not stimulate proteoglycan production.

20

Further evidence of the presence of TGF- $\beta$  was obtained by using antiserum raised against a synthetic peptide (TGF- $\beta$  78-109) from TGF- $\beta$ . This antiserum was added to the conditioned media taken from glomerular cultures on days 4 25 and 7 following ATS injection. The TGF- $\beta$  antiserum blocked the ability of the conditioned media to stimulate proteoglycan production (Figure 11). Proteoglycan production in mesangial cells exposed to conditioned media from normal control glomeruli was also slightly reduced by 30 the antiserum. Preincubation with the immunizing TGF- $\beta$  synthetic peptide, abolished the blocking effect of the antiserum on the induction of proteoglycan synthesis by conditioned media from day 7 nephritic glomeruli (Figure 12). In separate experiments, the TGF- $\beta$  antiserum blocked 35 the induction of proteoglycan synthesis, following addition of exogenous TGF- $\beta$  to cultured mesangial cells; this effect was reversed after addition of the immunizing peptide,

which also had no effect on proteoglycan induction when added to the condition media.

EXAMPLE V

5 MOLECULAR IDENTIFICATION OF GLOMERULAR PROTEOGLYCANS

The proteoglycans present in the glomerular cultures were identified with antibodies and an enzyme digestion. Labeled condition media from the day 7 glomerular cultures 10 was digested with specific enzymes or conditioned media after biosynthetic labeling as described in Example II. Samples for SDS-PAGE were mixed with sample buffer containing 3% SDS, 1 mM phenylmethylsulfonyl fluoride and 10%  $\beta$  mercaptoethanol and heated for 5 minutes at 100°C as 15 described above. Aliquots (20  $\mu$ l) were equally applied to 4-12% gradient gels (Novex, Encinitas, CA). Molecular size markers were from Pharmacia (Uppsala, Sweden). Fluorography was performed by incubating gels in Enlightning (New England Nuclear). Typical exposure times 20 for  $^{35}$ S sulfate were 3 to 5 days. Fluorograms were scanned with an Ultrascan XL Enhanced Laser Densitometer (Pharmacia) to compare and quantitate the relative intensities and mobilities of the proteoglycan bands. The results showed that the induced small proteoglycans were 25 fully sensitive to chondroitinase ABC and partially degraded by chondroitinase AC which indicates the presence of chondroitin/dermatan sulfate glycosaminoglycan chains (Figure 13). Immunoprecipitation of the same medium with specific antibodies, identified the 220 kD band as biglycan 30 and the 120 kD band as decorin (Figure 14). The immunoprecipitation was performed as described in Example II and the samples analyzed by SDS-PAGE as described above.

The proteoglycans produced by the cultured mesangial 35 cells in response to the conditioned media were identified as biglycan and decorin. These results are the same as observed following addition of exogenous TGF- $\beta$  to normal

rat mesangial cells in culture as described in Example II. The slight cross-reactivity of the anti-biglycan and decorin peptide antibodies seen in Figure 14 is likely to be due to the closely related sequences of the two core 5 proteins Fisher et al., J. Biol. Chem. 264:4571-4576 (1989), which is incorporated herein by reference).

EXAMPLE VI

DETECTION OF GLOMERULAR CELLS SYNTHESIZING TGF- $\beta$

10

Anti-LC is an antibody made against a synthetic peptide from TGF- $\beta$  that reacts with cells thought to be synthesizing TGF- $\beta$  (Flanders et al. Biochemistry 27: 739 (1988)). Anti-LC was used to detect TGF- $\beta$  production by 15 glomerular cells throughout the 28 day course of glomerulonephritis induced by ATS. Staining of glomeruli from normal control rats with this antibody showed an average of about 20 cells per glomerulus that were positive. In glomeruli from nephritic animals, the number 20 of glomerular cells stained by anti-LC was unchanged on day 4 but doubled on day 7, the peak of glomerular proteoglycan production. The temporal pattern of the increase in the number of cells positive for TGF- $\beta$  roughly parallels that 25 of proteoglycan production by glomeruli and the conditioned media (Figure 15). Figure 16A shows a representative anti-LC staining pattern of a glomerulus from a normal control animal compared to that of an animal 7 days after ATS injection (Figure 16B).

30

EXAMPLE VII

INHIBITION OF PROTEOGLYCAN SYNTHESIS IN NEPHRITIC GLOMERULI WITH ANTI-TGF- $\beta$  ANTIBODIES

Nephritis was induced in rats with a single injection 35 of ATS and the rats were then treated with either injections of anti-TGF- $\beta$  (78-109) or normal rabbit serum as a control. Ten animals were used in each group in three

different experiments. Figure 17 shows a comparison of representative glomeruli from kidneys of treated and control animals. The glomeruli have expanded less and contain less extracellular matrix in the anti-TGF- $\beta$ -treated 5 group than in the normal rabbit serum controls. Biochemical analysis showed that proteoglycan production by glomerular cells, which is high in the cells from the injured kidneys was suppressed by anti-TGF- $\beta$  (Figure 18). Scanning of the gel bands in Figure 18 and from other 10 similar experiments indicated that the suppression of this measure of the disease process was about 50 to 65%. These results show that the disease was substantially attenuated by the anti-TGF- $\beta$  treatment.

15 To gain information on the mechanism of the anti-TGF- $\beta$  effect, the level of TGF- $\beta$  mRNA was examined in the kidneys of the treated and control rats. TGF- $\beta$  can stimulate its own production. Therefore, an agent that 20 inhibits the activity of TGF- $\beta$  can also reduce its synthesis. The mRNA analysis revealed elevated levels of TGF- $\beta$  mRNA in the nephritis rats including the anti-TGF- $\beta$  treated animals. These results suggest that the antibody interrupted an paracrine loop of TGF- $\beta$  activity.

25

## EXAMPLE VIII

INHIBITION OF PROTEOGLYCAN SYNTHESIS WITH AN  
ARG-GLY-ASP CONTAINING PEPTIDE

30 Rat mesangial cells were grown to subconfluence in 6-well multiplates. The culture conditions and experimental protocol were as described in Example I. The cultures were made serum free for 24 hours and TGF- $\beta_1$  was added at 25 ng/ml along with Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) at 0.3, 0.1, 0.03, 0.01 or 0.003 mg/ml, or Gly-Arg-Gly-Glu-Ser-Pro 35 (GRGESP) at 0.3 mg/ml. The peptides were synthesized as described in Pierschbacher and Ruoslahti, J. Bio. Chem., 292:1794-1798 (1987) which is incorporated by reference

herein, on an Applied Biosystems Peptide Synthesizer according to the manufacturer's protocol. Thirty hours later the cultures were metabolically labeled with  $^{35}\text{S}$ -sulfate and 18 hours afterward the conditioned media were 5 analyzed by SDS-PAGE with fluorography. The fluorograms were scanned with a laser densitometer and the following represent relative densitometric units for the proteoglycan bands. Control 1.9, TGF- $\beta_1$  4.5, TGF- $\beta_1$ +GRGDS 0.3 mg/ml, 1.3, 0.1 mg/ml. 2.4, 0.03 mg/ml, 2.6, 0.01 mg/ml, 3.9, 10 0.003 mg/ml, 4.0 and GRGES 0.3 mg/ml, 4.3.

These data show a dose response effect of higher doses of GRGDSP causing inhibition of the TGF- $\beta_1$  induced proteoglycan production with no effect of the control 15 peptide GRGES.

#### EXAMPLE IX

#### DIFFERENTIAL EFFECTS OF TRANSFORMING GROWTH FACTOR B ON EXTRACELLULAR MATRIX PRODUCTION BY MESANGIAL CELLS AND GLOMERULAR EPITHELIAL CELLS

##### Methods

##### Growth Factors and Antibodies

25 Porcine TGF- $\beta_1$  was obtained from R&D Systems, Inc. (Minneapolis, MN). Human platelet-derived growth factor (PDGF) and recombinant human interleukin-1 (IL-1), alpha and beta, were from Collaborative Research, Inc. (Bedford, MA), recombinant human tumor necrosis factor (TNF) was from 30 Amgen (Thousand Oaks, CA). Goat anti-human type I and III collagen antibodies were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Rabbit anti-mouse fibronectin and types I, III, IV and VI collagen and production of anti-rat laminin antibodies have been 35 described. Briefly, rabbit polyclonal antibodies were produced by injecting New Zealand rabbits with each purified protein emulsified in Complete Freund's Adjuvant

subcutaneously. The initial immunization was followed three weeks later with sequential weekly injections of purified protein emulsified in Incomplete Freund's Adjuvant intramuscularly. The secondary injections were accompanied 5 by a bleed of 50 ml from an ear artery. The blood was coagulated and the sera was collected by centrifugation. Monoclonal antibody to rat Thy-1 was obtained from Accurate Chemicals (Westbury, N.Y.) and goat anti-human factor VIII from Miles Laboratory Inc. (Kankokee, IL).

10

Rabbit anti-rat FX1A antibody was produced by immunization with FX1A prepared from the kidney of Sprague-Dawley rats according to the method of Edgington. Briefly, rabbits were immunized as described above with preparation 15 enriched in brush border of the proximal renal tubules that was isolated by ultracentrifugation. FITC and alkaline phosphatase labeled or anti-rabbit IgG and anti-goat IgG were obtained from Cappel (Melvern, PA).

## 20 Cell Culture

Glomerular epithelial cells and mesangial cells were obtained from outgrowth of intact glomeruli obtained from 6 to 8 week old Sprague-Dawley rats. Our technique for 25 isolation and culture of mesangial cells was performed as follows.

Male Sprague/Dawley rats (100-140 grams, 2 rats per isolation) were anesthetized using ketamine (10 mg/100 g 30 rat) and Rompum (0.5 mg/rat) and sterilized using betadine. The kidneys were exposed surgically and perfused using phosphate-buffered saline. The kidneys were then removed and placed in sterilized iced PBS.

35 The kidneys were bisected and the cortex was removed surgically. The remaining tissue was minced into 1 mm<sup>3</sup> pieces using a scalpel. The minced kidney tissue was then

passed through a series of sieves (149 mesh and 105 mesh) using continuous washing with PBS. The material passing through the 105 mesh sieve is then collected on a 74 mesh sieve. This represents the glomerular fraction.

5

This fraction was centrifuged for 10 minutes in a clinical centrifuge and the pellet was resuspended in RPMI media containing 20% fetal bovine serum, 0.66 units/ml insulin, and antibiotics. The glomeruli were plated at 2 10 kidney equivalents in 12 ml media in a T 75 flask. The mesangial cells grow out from the attached glomeruli after approximately 3 weeks of culture. Fresh media is added every 3-4 days during this culture period.

15 To isolate epithelial cells, intact glomeruli were placed in flasks coated with vitrogen (Collagen Corporation, Palo Alto, CA). The growth medium was RPMI 1640 (Cell-Gro, Washington, D.C.) supplemented with 20% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, 20 UT), 50 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.66 U/ml insulin and 300 mg/ml L-glutamine. After 7 days, outgrowing cells were detached with 0.025% trypsin-0.5M EDTA (Flow Labs, McLean, VA). Glomerular debris was removed by passing the material through a 30-mesh screen. 25 A highly enriched epithelial cell population was produced by using an indirect panning technique. Specifically, glomeruli were isolated as described above and plated on tissue culture dishes pre-coated with rat tail collagen type I at 0.15 mg/ml. The dishes were coated for 1 hour 30 at room temperature, then washed 2 times with PBS. After 1 week of culture, the outgrowth of cells (primarily epithelial cells) and glomeruli were trypsinized using 0.05% Trypsin-0.53 mM EDTA. The cell suspension was passed through a 30 mesh to remove glomerular pieces. The 35 remaining cell suspension was plated on a dish previously coated with a monoclonal antibody against the Thy 1 antigen. Plated was coated with antibody (approximately

100 µg/ml) at 4°C for 24 hours. The incubation of cells on the anti-Thy 1-coated dish was for 1 hour at 37°C. The unbound cells were collected and replated. Only mesangial cells will bind to the Thy 1 antibody because they express 5 the antigen, whereas the epithelial cells do not and thus remain in suspension. This procedure preferentially eliminated mesangial cells by binding to plates coated with anti-Thy-1 antibody, mesangial cells but not epithelial possess a Thy-1 like epitope on their surface. Glomerular 10 cells were added to 60 X 15 mm polystyrene plates, which were precoated with mouse anti-Thy-1 antibody by 12 hour incubation at 4°C. Nonadherent cells were removed and placed in 6-well plates precoated with laminin (Collaborative Research, Bedford, MA).

15

Glomerular epithelial cells were identified by: 1) characteristic polygonal morphology, 2) uniform staining with antibody to FX1A (Heymann antigen), 3) sensitivity to aminonucleoside of puromycin, and 4) no staining with anti- 20 Thy-1 or anti-Factor VIII antibody. Contamination of glomerular epithelial cells by proximal tubular cells was examined by alkaline phosphatase staining, and less than 2% of the cells were alkaline phosphatase positive. In contrast, mesangial cells were identified by 1) typical 25 stellate appearance, 2) uniform staining with anti-Thy-1 antibody, 3) no staining with anti-FX1A or anti-Factor VIII antibody, and 4) no sensitivity to aminonucleoside of puromycin.

### 30 Biosynthetic Radiolabeling

Glomerular epithelial and mesangial cells were added to regular or laminin-coated 6-well multiwell plates at a concentration of 5 X 10<sup>5</sup> cells per well and cultured in 35 serum-free RPMI 1640 medium for 24 hours to arrest cell proliferation. Non-adherent cells were removed by washing with phosphate buffered saline (PBS). Serum and

antibiotic-free RPMI 1640 was added as a low sulfate medium for  $^{35}\text{S}$  sulfate labeling and RPMI 1640 without methionine (Flow Labs, McLean, VA) for  $^{35}\text{S}$  methionine labeling. Growth factors were added to the media for 48 hours at 5 concentrations used in the above Examples. The concentrations chosen were: TGF- $\beta$  (25 ng/ml), PDGF (2 U/ml), IL-1 $\alpha$  (5 U/ml), IL-1 $\beta$  (5 U/ml) and TNF (500 U/ml). Eighteen hours prior to termination of the experiment,  $^{35}\text{S}$  methionine (100  $\mu\text{Ci}/\text{ml}$ ), to label proteins or  $^{35}\text{S}$  sulfate 10 (200  $\mu\text{Ci}/\text{ml}$ ) to label proteoglycans were added to the cultures.

Isotopes were purchased from New England Nuclear (Boston, MA). Conditioned media and cell layers were 15 harvested and processed as described above. Proliferation of cells was examined by the uptake of  $^3\text{H}$ -thymidine. Cells were added to laminin-coated 12 well plates at a concentration of  $2 \times 10^5/\text{well}$ . After 24 hours in serum free medium, 25  $\mu\text{l}$  of a 10  $\mu\text{Ci}/\text{ml}$  solution of  $^3\text{H}$ -thymidine was 20 added to each well. Incubation was carried out for 24 hours. The culture medium was discarded, cell layers were washed 2 times with 5% TCA solution, and incubated for 15 minutes with 700  $\mu\text{l}$  of 6 N HCl solution. Incorporation of  $^3\text{H}$ -thymidine was measured by counting each cell layer in a 25 liquid scintillation counter (Beckman, Irvine, CA) and corrected with protein contents of each cell layer which were measured by a BCA protein assay kit (Pierce, Rockford, IL).

### 30 Identification of Matrix Molecules

Matrix glycoproteins were identified by immunoprecipitation and proteoglycan by enzyme digestion as described above.

35

Large proteoglycans were characterized by gel filtration.  $^{35}\text{S}$  sulfate labeled fractions from a sepharose

CL-6B column were dialyzed against distilled water and lyophilized. Samples were dissolved in 2 ml of 0.1M sodium acetate 0.1M tris, pH 7.3, containing protease inhibitors described above. Then 0.2 ml of chondroitinase ABC 5 solution (1.25 units/ml) was added to 1.8 ml of sample digestion carried out for 24 hours at 37°C. Samples were then fractionated on a CL-6B column, dialyzed against distilled water, lyophilized and treated with nitrous acid under low pH (1.0) conditions. After treatment with 10 nitrous acid, samples were rechromatographed on a CL-6B column.

To quantitate the increased production of proteoglycans induced by TGF- $\beta$ , three ml of conditioned 15 medium were placed on a sepharose CL-6B column (1.3 x 100 cm). The column was eluted with 4M urea, 0.15M NaCl, 50 mM Tris HCl, pH 7.2, 0.1% (v/V) Triton X-100 at a flow rate of 20 ml/hour, and fractions of 3 ml/tube were collected. The radioactivity in each effluent fraction was determined by 20 a Beckman LS 2800 liquid scintillation counter.

#### Electrophoretic Technique

Samples were analyzed by SDS-PAGE with fluorography 25 and densitometry as described above.

#### Results

This example demonstrates that TGF- $\beta$  regulates the 30 production of biglycan, fibronectin and type IV collagen by the epithelial cells, while TGF- $\beta$  only affects proteoglycan production by cultured mesangial cells. The biosynthetic profile of matrix production by epithelial cells, regulated by TGF- $\beta$ , is thus different from that of mesangial cells. 35 These results demonstrate that release of TGF- $\beta$  in the glomerulus leads to mesangial matrix accumulation and an increase in production of matrix molecules that form the

glomerular basement membrane. The results are summarized in Table I.

5

TABLE I  
TGF- $\beta$  Induction of Extracellular Matrix by  
Glomerular Cells in Culture

	<u>Matrix Components</u>	<u>Mesangial Cell</u>		<u>Epithelial Cell</u>	
		<u>Medium</u>	<u>Monolayer</u>	<u>Medium</u>	<u>Monolayer</u>
Proteoglycans					
	Biglycan	↑↑↑↑	↑	↑↑↑↑	0
15	Decorin	↑↑↑↑	↑	↑	0
	Heparan Sulfate	0	0	0	0
20	Fibronectin	0	↑	↑↑↑↑	↑↑↑↑
	Laminin	0	0	↑	↑↑
Collagen					
25	Type I	0	0	NP	NP
	Type III	0	0	NP	NP
30	Type IV	0	0	↑↑↑	↑↑↑
	Type VI	0	0	NP	NP

35

↑ = induction, 0 = no effect, NP = component not produced

Although the invention has been described with reference to the presently-preferred embodiment, it should 40 be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

## WE CLAIM:

1. A method for treating pathologies characterized by an accumulation of extracellular matrix in a tissue, comprising contacting said tissue with an agent which suppresses the extracellular matrix producing activity of TGF- $\beta$ .

2. The method of claim 1 wherein said agent is anti-TGF- $\beta$  antibody.

3. The method of claim 1 wherein said agent is PDGF.

4. The method of claim 1 wherein said agent is an Arg-Gly-Asp-containing peptide.

5. The method of claim 1 wherein said pathologies are selected from the group consisting of glomerulonephritis, adult respiratory distress syndrome, cirrhosis of the liver, fibrotic cancer, fibrosis of the lungs, arteriosclerosis, post myocardial infarction, cardiac fibrosis, post-angioplasty restenosis, renal interstitial fibrosis and scarring.

6. A method of inhibiting the accumulation of extracellular matrix in a tissue, comprising suppressing the activity of TGF- $\beta$  in the tissue.

7. The method of claim 6 wherein suppressing the activity of TGF- $\beta$  comprises contacting the tissue with anti-TGF- $\beta$  antibodies.

8. The method of claim 6 wherein suppressing the activity of TGF- $\beta$  comprises contacting the tissue with PDGF.

9. The method of claim 6 wherein suppressing the activity of TGF- $\beta$  comprises contacting the tissue with a Arg-Gly-Asp-containing peptide.

10. The method of claim 6 wherein said tissue is comprised of cells selected from the group consisting of kidney, lung, liver and skin cells.

11. A method of detecting the presence of pathologies of a tissue characterized by an excessive accumulation of extracellular matrix components, comprising determining the level of TGF- $\beta$  in said tissue and comparing the level of 5 TGF- $\beta$  in said tissue to the level of TGF- $\beta$  in normal tissues, an elevated level of TGF- $\beta$  in said tissue being indicative of such pathologies.

12. The method of claim 11, wherein said pathologies are selected from the group consisting of glomerulonephritis, adult respiratory distress syndrome, cirrhosis of the liver, fibrotic cancer, fibrosis of the lungs, arteriosclerosis, post myocardial infarction, cardiac fibrosis, post-angioplasty restenosis, renal interstitial fibrosis and scarring.

13. A method of decreasing the production of an extracellular matrix component by a cell which produces an extracellular matrix component comprising decreasing the amount or inhibiting the activity of TGF- $\beta$  to which said cell is exposed.

14. The method of claim 13 wherein said cell is a mesangial cell.

15. The method of claim 13, wherein said cell is an epithelial cell.

16. The method of claim 13, wherein said extracellular matrix component is a proteoglycan.

17. The method of claim 16, wherein said proteoglycan is selected from the group consisting of biglycan and decorin.

18. The method of claim 13, wherein said extracellular matrix component is selected from the group consisting of fibronectin, laminin and type IV collagen.

19. An antibody which inhibits the proteoglycan stimulating activity of TGF- $\beta$  having an affinity of about  $10^8$  or greater and a titer of about 1:30,000 or greater as measured by radio immunoassay.

20. The antibody of claim 19, produced by immunizing an animal with a linear peptide from TGF- $\beta$ .

21. A cell which produces the antibody of claim 19.

1/11



FIG. 1

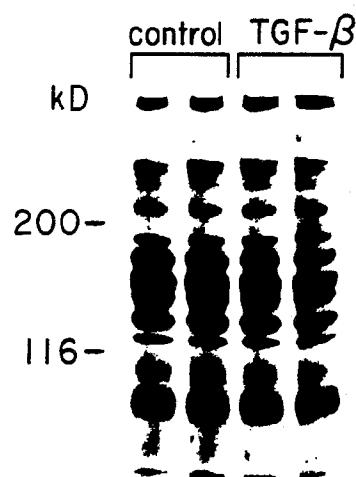


FIG. 2

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2 / 11

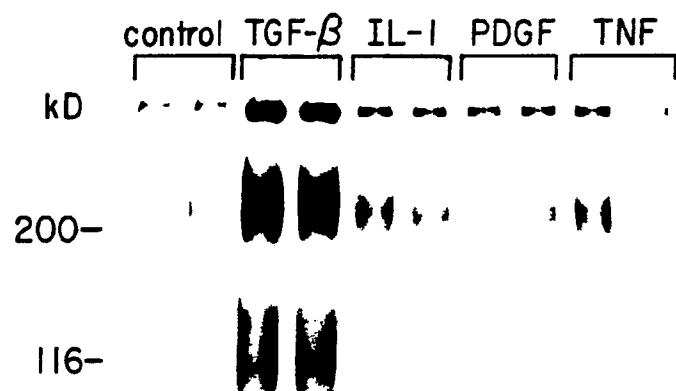


FIG. 3



FIG. 4

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3 / 11

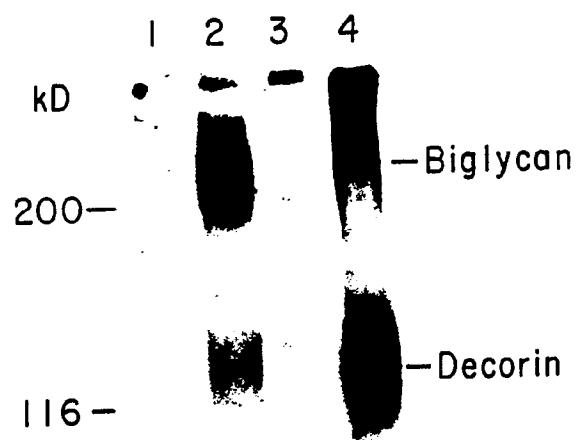


FIG. 5

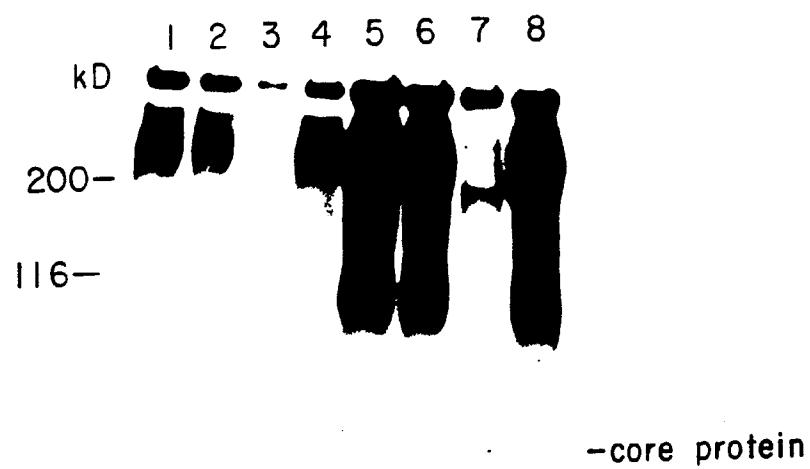
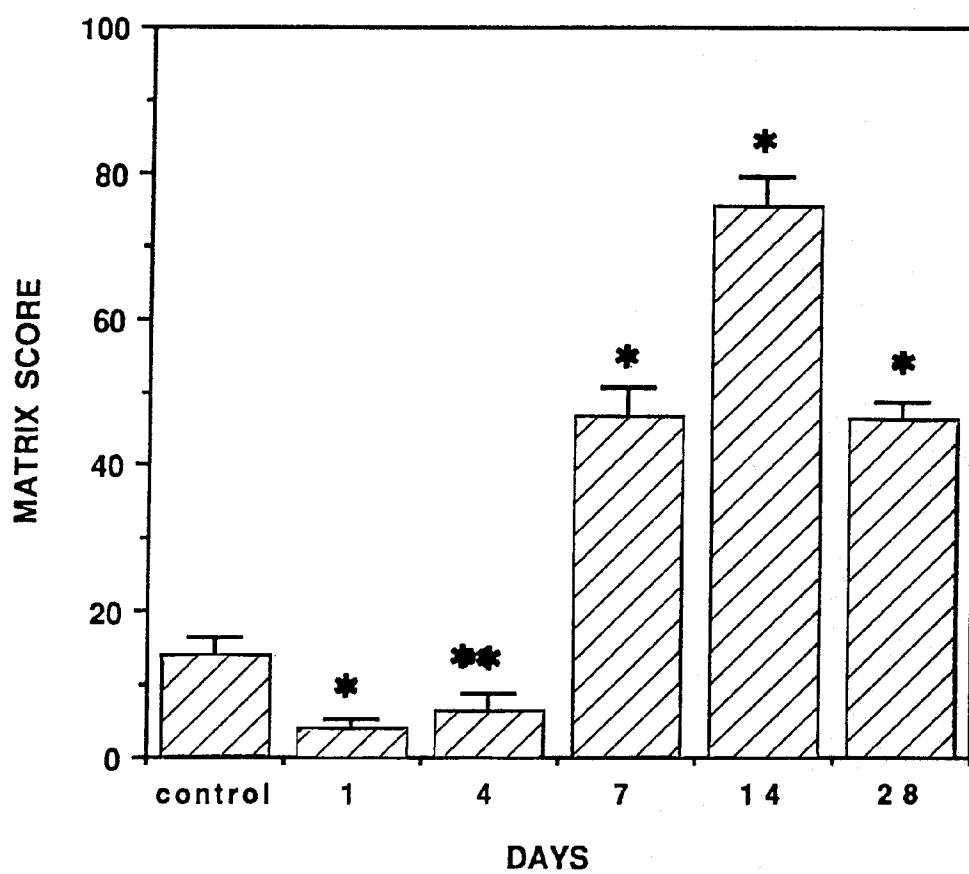


FIG. 6

4 / 11

FIG. 7



SUBSTITUTE SHEET

5 / 11



FIG. 8a

SUBSTITUTE SHEET

6 / 11



FIG. 8b

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7/11  
DAYS  
0 1 4 7 14 28

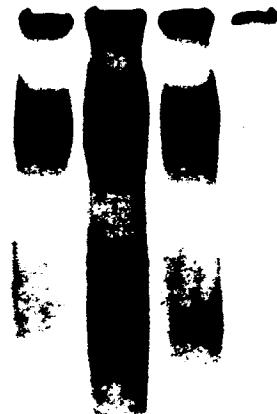


FIG. 9

DAYS  
0 1 4 7 14 28



FIG. 10

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8 / 11



FIG. 12

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9 / 11

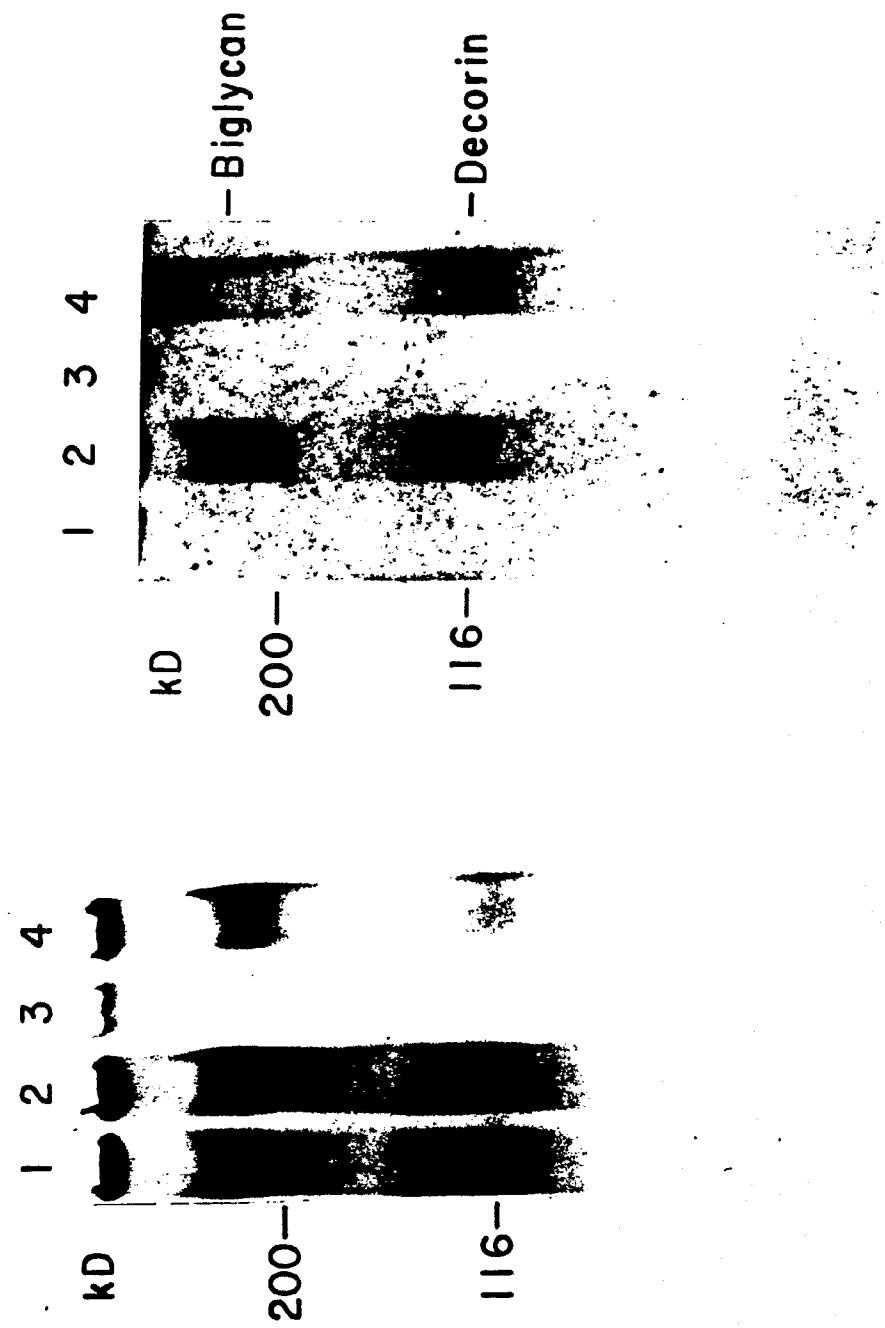


FIG. 13

FIG. 14

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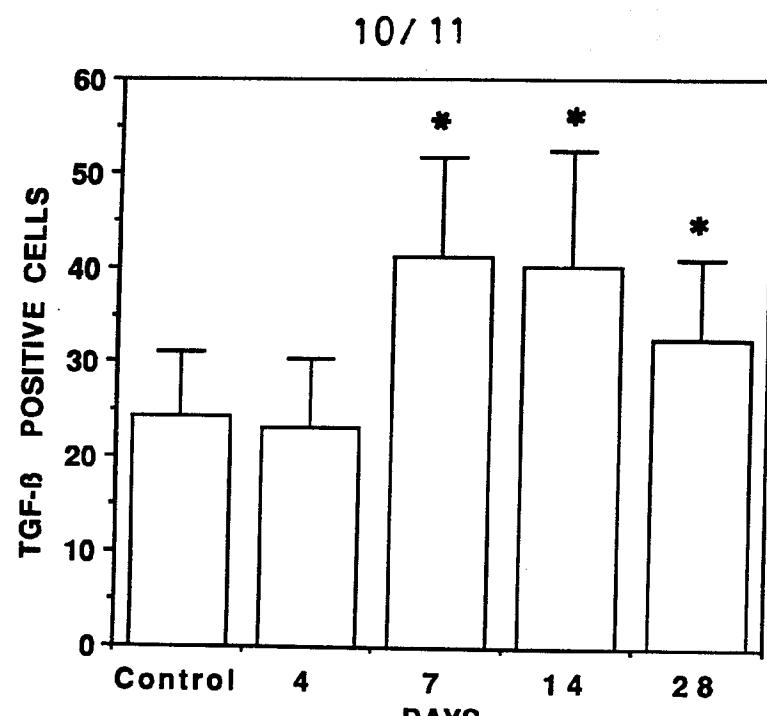


FIG. 15



FIG. 16a



FIG. 16b

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11 / 11

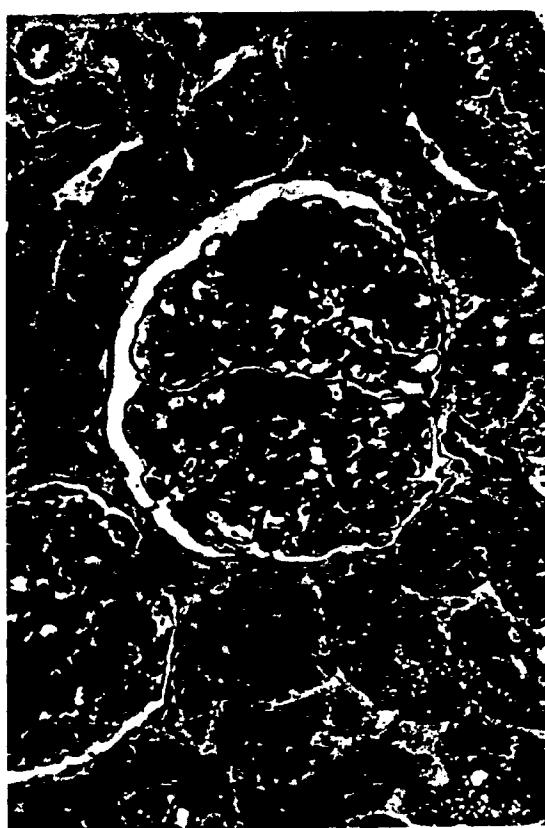


FIG. 17a

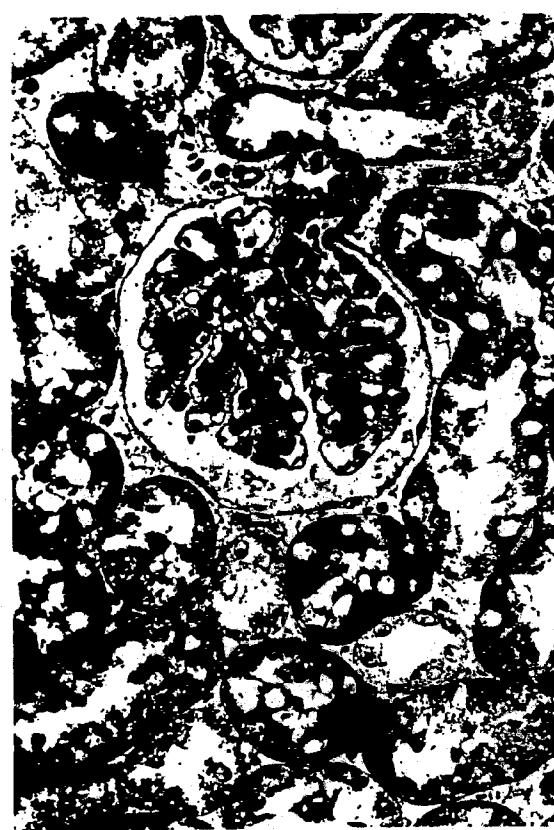
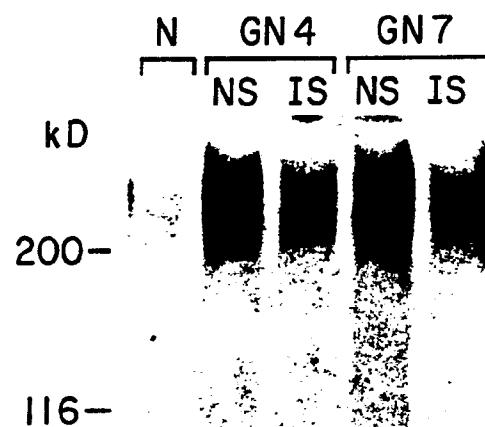


FIG. 17b

FIG. 18  
SUBSTITUTE SHEET

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/05566

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC<sup>5</sup>: A 61 K 39/395, A 61 K 37/02, A 61 K 37/36,  
G 01 N 33/53

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>7</sup>

Classification System	Classification Symbols
IPC <sup>5</sup>	A 61 K, C 12 P, C 07 K
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	Journal of Clinical Investigation, vol. 83, May 1989, The American Society for Clinical Investigation, Inc., T.B. Connor, Jr. et al.: "Correlation of Fibrosis and transforming growth factor-beta type 2 levels in the eye", pages 1661-1666, see the whole article --	11-12
X	The Journal of Biological Chemistry, vol. 261, no. 9, 25 March 1986, The American Society of Biological Chemists, Inc., R.A. Ignatz et al.: "Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix", pages 4337-4345, see the whole article	6,9-10,13,18

\* Special categories of cited documents: <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

5th February 1991

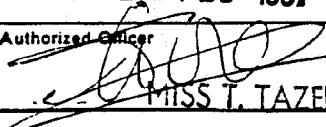
Date of Mailing of this International Search Report

22 FEB 1991

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

  
MISS T. TAZELAAR

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Proceedings of the National Academy of Sciences USA, vol. 83, June 1986, A.B. Roberts et al.: "Transforming growth factor type beta: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro", pages 4167-4171, see the whole article  --	6,7,9-10, 13,16-18
X	Biochemistry, vol. 27, 1988, American Chemical Society, K.C. Flanders et al.: "Antibodies to peptide determinants in transforming growth factor beta and their applications", pages 739-746, see the whole article  --	19-21
X,P	Nature, vol. 346, 26 July 1990, W.A. Border et al.: "Suppression of experimental glomerulonephritis by antiserum against transforming growth factor betal", pages 371-374, see the whole article  --	6-7,10-21
A	WO, A, 88/03151 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 5 May 1988 see page 31, line 15 - page 32, line 8  --	6-18
A	Proceedings of the National Academy of Sciences USA, vol. 84, August 1987, J.-K. Chen et al.: "Transforming growth factor type beta specifically stimulates synthesis of proteoglycan in human adult arterial smooth muscle cells", pages 2587-2591, see the whole article  --	6-18

. / .

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X,O      Meeting of the American Society of Nephrology, San Antonio, Texas 11-14 December 1988,  
 W. Border et al.: "Transforming growth factor beta TGF beta uniquely regulates production and structure of glomerular extracellular matrix proteoglycans", & Kidney Int. 35(1).1989, 341 see abstract

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6,7,10,13-18

V.  OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.  Claim numbers .....x...., because they relate to subject matter not required to be searched by this Authority, namely:

Claims searched incompletely: 6-10,13-18

Claims not searched: 1-5

See PCT-Rule 39.1(iv):methods for treatment of the human or animal body by surgery or therapy as well as diagnostic methods

2.  Claim numbers .....x....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.  Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.  OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.  As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

The additional search fees were accompanied by applicant's protest.

No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.

US 9005566  
SA 41315

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 19/02/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A- 8803151	05-05-88	AU-A-	8239987	25-05-88